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Study the symbiotic crude oil-degrading bacteria in the mussel *Mactra stultorum* collected from the Persian Gulf

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ABSTRACT

Symbiotic associations are complex partnerships that can lead to new metabolic capabilities and the establishment of novel organisms. The diversity of these associations is very broad and there are still many mysteries about the origin and the exact relationship between the organisms that are involved in a symbiosis. The aim of the present study is to find symbiotic crude-oil degrading bacteria in the mussels that collected from the Persian Gulf. Fifteen crude-oil degrading bacteria were isolated from *Mactra stultorum* mussel that collected from oil contaminated area at Persian Gulf. According to high growth rate on crude oil five strains were selected from 15 isolated strains for more study. Determination of the nucleotide sequence of the gene encoding 16S rRNA show that these isolated strains belong to: *Alcanivorax dieselolei* strain BHA25, *Idiomarina baltica* strain BHA28, *A. dieselolei* strain BHA30, *Alcanivorax* sp. strain BHA32 and *Vibrio azureus* strain BHA36. Analysis of remaining of crude oil by Gas Chromatography (GC) confirmed that these strains can degrade: 64%, 63%, 71%, 58% and 75% of crude oil respectively.

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1. Introduction

Oil contaminated wastewater comes from variety of sources into marine environment such as crude oil production, oil refinery, petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents (Head et al., 2006). Oily wastewater contains toxic substances such as phenols, petroleum hydrocarbons and poly aromatic hydrocarbons (PAH) which are inhibitory to plant and animal growth, equally mutagenic and carcinogenic to human being. Similarly, oily wastewater contains high oil content, chemical oxygen demand (COD) and color (Yakimov et al., 2007; Ghanavati et al., 2008).

Toxicity of crude oil includes liver necrosis, congestion of the liver, fat degeneration, and dissociation of hepatocytes. Birds and animals in oil-contaminated area are found to have black emulsion in the digestive tract with a petroleum odor. This leads to decrease in the absorption of nutrients and finally leads to death of these birds and animals due to rupture of capillaries and hemorrhage (Hassanshahian et al., 2010).

Increasing attention has been paid for developing and implementing innovative technology for cleaning up these contaminants. Bioremediation methods are currently receiving favorable publicity as promising environmental friendly treatment technologies for the remediation of hydrocarbons (Hasanshahian and Emtiazi, 2008). Moreover, biological methods can have an edge over the physicochemical treatment regimes in removing spills as they offer cost effective *in situ* biodegradation of oil fractions by the microorganisms (Yakimov et al., 2007). Biostimulation, the use of microorganisms via addition of fertilizers to improve their population, or the direct addition of microorganisms (bioaugmentation) have been studied as means of remediation the harmful effect of crude oil pollution (Hassanshahian et al., 2012b). The discovery of indigenous marine bacteria specialized in the degradation of hydrocarbons (Hydrocarbonoclastic Bacteria, HCB) has highlighted the major role that bacteria play in the marine natural cleansing processes and offered new perspectives for bioremediation (Hassanshahian et al., 2014b). Marine organisms can take up contaminants from bottom sediments, suspended particulate material and food sources (Head et al., 2006; Hassanshahian et al., 2012a).

Bivalves are ecologically important members of coastal and estuarine communities. Bivalves filter large volumes of water to meet their food requirements and accumulate dissolved oil components and particles containing hydrocarbons present in oil polluted water columns. Bivalves can be filter and concentrate bacteria could contribute in reducing the bacterial concentration in seawater. Bacterial concentrations were higher in the mussel samples compared to the corresponding seawater throughout the year, thus playing an important role in the process of bioremediation of the marine environment (Zhou et al., 2014; Bayat et al., 2015).

The relationship between heterotrophic and hydrocarbon-degrading bacteria and benthic filter feeders may be functionally important to aquatic ecosystems especially polluted ones (Wang and Chow, 2002). The aim of the present study is study some bacterial strains isolated from mussels that collected from oil-polluted sites in the Persian Gulf also, define which of the symbiotic bacteria with bivalve involved in crude oil biodegradation.

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2. Materials and methods

2.1. Sampling

For isolation of crude oil degrading bacteria mussel samples were collected by hand (depth range 0.5-5 m) from Terminal of Bandar Abbas at Persian Gulf (47° 30, N; 49° 15, E). The samples were collected into sterile jars, placed on ice, and immediately transported to the laboratory for further analysis. Shell of mussels removed with a sterile knife then gill and other tissue of sample washed with sterile seawater. Then the tissue was homogenized in sterile seawater. Finally bivalve solution was used for subsequent studies. Fig. 1 shows the map of sampling zones.

2.2. Isolation and selection of crude-oil degrading bacteria

The ONR7a medium supplemented with 1% (*v*/v) of crude-oil (Iranian light crude oil) as sole carbon source and energy used for isolation of crude oil degrading bacteria. ONR7a contained (per liter of distilled water) 40 g of NaCl, 11.18 g of $MgCl_2.6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2.2H_2O$, 1.3 g of TAPSO {3-[N tris(hydroxymethyl) methylamino]-2 hydroxypropanesulfonica cid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HPO_4.7H_20$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCl_2.6H_2O$, 2.6 mg of NaF and 2 mg of $FeCl_2.4H_20$. For solid media, Bacterial Agar (15 g/l) was added to the solution (Cappello et al., 2012a, 2012b; Hassanshahian et al., 2013).

Solution of mussel (5 ml) were added to Erlenmeyer flasks containing 100 ml of medium and the flasks were incubated for 7 days at 30 °C on rotary shaker (180 rpm, INFORS AG). Then 5 ml were transported to fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out, and phenotypically different colonies on ONR7a agar were purified. Phenotypically different colonies obtained from the plates were transferred to fresh medium with and without crude oil to eliminate autotrophs and agar utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil were stored in stock media with glycerol at −20 °C for further characterization (Cappello et al., 2012a, 2012b; Hassanshahian et al., 2014a).

2.3. Identification of the isolates

2.3.1. Biochemical characterization

The following characteristics were determined according to the “Bergey's Manual of Determinative Bacteriology: the Gram stain, motility, starch hydrolysis, indole, H2S production, catalase and oxidase, oxidation/fermentation, reduction of nitrate, Growth and acidification of carbohydrates tests were performed (Holt et al., 1998).

2.3.2. Molecular identification

Analysis of 16S rRNA was performed to taxonomically characterize of the isolated strains. Total DNA extraction of bacterial strains was performed with the CTAB method. PCR amplification of 16S rRNA genes was performed using the general bacteria primer 27F (5-AGAG TTTGATCCTGGCTCAG-3) and universal reverse primer 1492R (5-TACGYTACCTTGTTACGACTT-3). The amplification reaction was performed in a total volume of 25 μl consisting, 2 mM $MgCl_2$ (1 μl), 10× PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 μl), 2 mM each dNTP (2 μl), 0.15 mM each primer (1 μl), 1 U (0.5 μl) taq DNA polymerase (Qiagen, Hilden, Germany) and 2 μl of template DNA (50 p). The distill water was added for remaining of reaction (15 μl). Amplification for

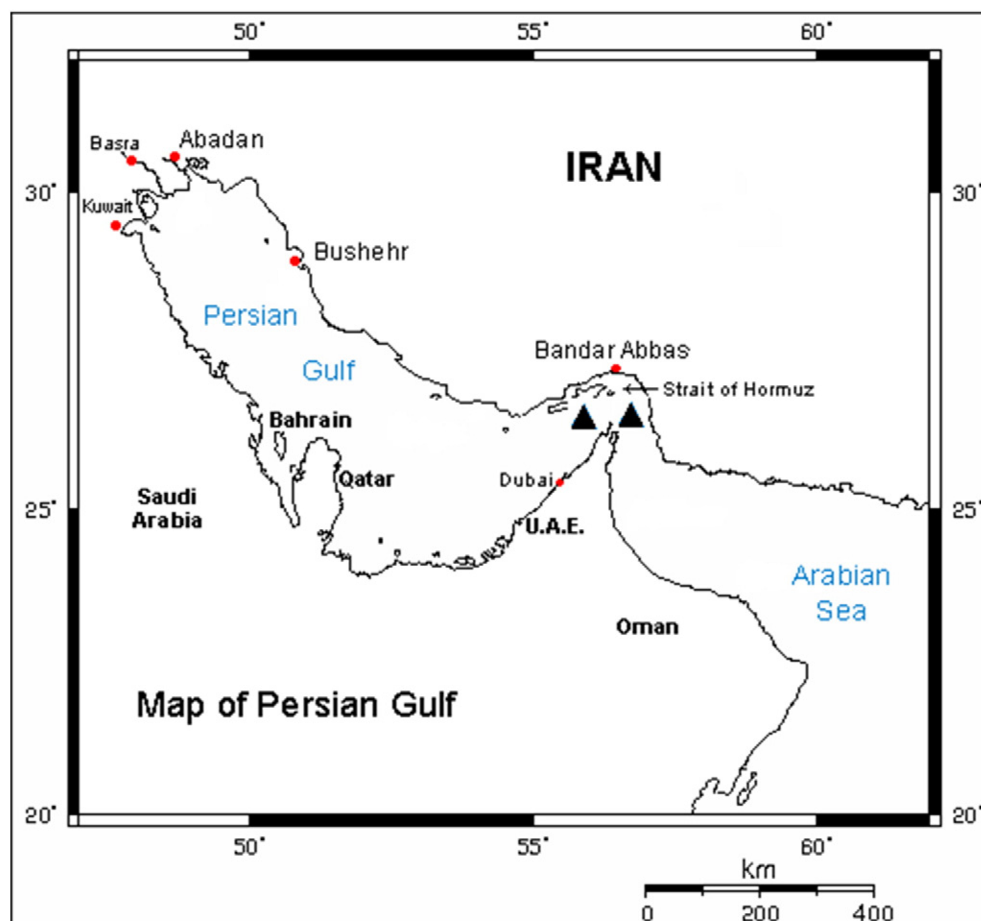

Fig. 1. The map of sampling zones.

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